Physiology & Behavior 122 (2013) 76–83

Contents lists available at ScienceDirect

Physiology & Behavior

journal homepage: www.elsevier.com/locate/phb

Lactational exposure to sulpiride: Assessment of maternal care and reproductive and behavioral parameters of male rat pups

Milene Leivas Vieira [a], Alice Hartmann dos Santos [a], Luiza Sienna Silva [a], Glaura Scantamburlo Alves Fernandes [b], Ana Carolina Inhasz Kiss [a], Estefânia Gastaldello Moreira [a], Suzana de Fátima Paccola Mesquita [b], Daniela Cristina Ceccatto Gerardin [a,*]

[a] Department of Physiological Sciences, State University of Londrina, 86051-980 Londrina, Paraná, Brazil
[b] Department of Biology, State University of Londrina, 86051-980 Londrina, Paraná, Brazil

HIGHLIGHTS

- Maternal exposure to SUL did not impair maternal behavior.
- SUL caused testicular damage and testes seem to be the main target organ at adulthood.
- No adverse effect was found in behavior parameters of male pups.

ARTICLE INFO

Article history:
Received 6 November 2012
Received in revised form 27 June 2013
Accepted 30 August 2013

Keywords:
Galactagogue
Dopaminergic receptor antagonist
Testis
Prostate

ABSTRACT

Dopaminergic receptor antagonists may be used as galactagogues because they increase serum prolactin (PRL) by counteracting the inhibitory influence of dopamine on PRL secretion. The antipsychotic drug sulpiride (SUL) is documented to be effective as a galactagogue, but it is transferred through milk to the neonates. The aim of the present study was to evaluate if maternal exposure to SUL during lactation could disrupt maternal care and/or male offspring reproductive development. The dams were treated daily (gavage) with SUL 2.5 mg/kg or 25 mg/kg during lactation. Maternal behavior was analyzed on lactational days 5 and 10. In offspring, reproductive and behavioral parameters were analyzed at different time points. SUL treatment did not impair maternal care, but caused testicular damage in male offspring. At postnatal day 90, a reduction in testis weight, volume of seminiferous tubule and histopathological alterations such as an increased percentage of abnormal seminiferous tubules were observed. Data shows that maternal exposure to SUL during lactation may impact the reproductive development of male rats and the testes seem to be the main target organ at adulthood.

1. Introduction

Breast milk production is a complex physiologic process involving physical and emotional factors and the interaction of multiple hormones. The most important of them is believed to be prolactin (PRL) [1]. Failure to provide sufficient breast milk in the first few postpartum days is a major cause of breastfeeding failure [2]. Because of this, both mothers and physicians have sought drugs to address this concern [1]. The available galactagogues are dopaminergic antagonists, which increase serum PRL by counteracting the inhibitory influence of dopamine (DA) on PRL secretion [3].

Sulpiride (SUL) is mainly marketed as an antipsychotic drug and is reported to selectively antagonize central dopaminergic receptors (D2, D3 and D4) [4]. Moreover, SUL has also been documented as an effective galactagogue [5–7] but it is important to consider that maternal use of this drug results in exposure of the progeny. Studies with humans [3,8,9] and rats [7] reported that SUL is excreted unaltered in milk [3,6–8,10,11] and, in humans, it is estimated that maternal exposure to 100 mg/kg of SUL daily would result in an average daily intake of 0.135 mg/kg in infant [11].

DA is involved in both the onset and the maintenance of maternal care [12–15]. The main nucleus that regulates this behavior is the medial preoptic area (MPOA) of hypothalamus [16–18]. Some aspects of the maternal behavior are regulated by the mesolimbic dopaminergic system in this area [18] and disruption of mother–pup interaction was reported after maternal exposure to dopaminergic antagonist [19,20].

Moreover, in rodents, maternal treatment during lactation with antipsychotic drugs such as risperidone [21] and SUL [7] resulted in neonatal hyperprolactinemia in the progeny. It is known that PRL plays an important role in the regulation of testicular function and participates in the regulation of growth and normal function of all tissues sensitive

* Corresponding author at: Department of Physiological Sciences, State University of Londrina—UEL, 86051-980 Londrina, Paraná, Brazil. Tel.: +55 43 3371 4307; fax: +55 43 3371 4467.
E-mail address: dcgerardin@uel.br (D.C.C. Gerardin).

0031-9384/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.physbeh.2013.08.027

to androgens [22]. So, drug-induced hyperprolactinemia in newborns may impact normal development of the reproductive system.

Based on these considerations, this study was carried out in rats in order to evaluate if maternal exposure to SUL during lactation could disrupt maternal care and/or male offspring's reproductive development.

2. Materials and methods

2.1. Animals and treatment

Male and female Wistar rats (85–90 days) from the colony of the State University of Londrina (UEL) were used as parental generation. They were kept in a controlled environment with temperature at 21 ± 2 °C; humidity of 55 ± 5%; 12 h light/dark cycle (lights on at 6:00 a.m.) and had free access to regular lab chow and tap water.

Rats were mated (2 females and 1 male per cage) and gestational day 0 was determined if there were sperm and estrus phase cells in vaginal smears. Dams were divided into three groups (20 dams/group):

- Control group (CON): dams received daily 0.20 ml of distilled water, by gavage, from post-natal day (PND) 0 to PND 21;
- SUL 2.5 mg group (SUL 2.5): dams received daily 2.5 mg/kg of SUL (Equilid™, Aventis, Brazil), by gavage, from PND 0 to PND 21;
- SUL 25 mg group (SUL 25): dams received daily 25 mg/kg of SUL (Equilid™, Aventis, Brazil), by gavage, from PND 0 to PND 21.

The dams were daily treated at 12:00–2:00 p.m. The drug was dissolved in distilled water immediately prior to the treatment.

In humans, the typical dosage for initiation of lactation is 50 mg 2 to 3 times daily [5,6,10,23], which would correspond to approximately 1.7 to 2.5 mg/kg. To address the possibility that rodents may be less sensitive to drugs than human, we have also chosen to evaluate the dose of 25 mg/kg, which is 10 times higher than the highest human dose employed as galactagogue.

At PND 0, all litters were weighed and on PND 1 they were culled to 8 pups. Whenever possible, an equal number of male and female pups were kept within the litter. Pups were weaned on PND 21 and housed in groups separated by gender and tests until the evaluations. The litter was the experimental unit (i.e. one male pup per litter was used for each evaluation at each time point). All animals had free access to water and regular lab chow (Nuvital™, Nuvilab, Brazil) and all animal procedures were approved by the UEL Ethics Committee for Animal Research (CEEA 26/11). The experimental design is represented in Fig. 1.

2.2. Parameters analyzed in dams

2.2.1. Maternal general toxicity

Maternal body weight and food intake were recorded weekly (PNDs 0, 7, 14 and 21) during lactation. Toxicity signs (e.g. lacrimation, piloerection, unusual respiratory pattern and tremors) were evaluated daily during treatment. On PND 21, dams were submitted to the open-field test [24] with two objectives: 1) to evaluate their behavior outside the home-cage; 2) to ensure that motor function was not compromised and would not have interfered with maternal care since that mesolimbic and striatal dopaminergic pathways influence motor function [25]. Briefly, the apparatus consisted of a circular surface of wood (60 cm of diameter) surrounded by a wall. The surface was painted white and divided into similar parts. Each animal was placed individually in the center of the arena and the following variables were recorded during a 3-min session: ambulation (count of floor units entered with the four paws), rearing (count of times that the animal stood on its hind legs), and grooming (time, in seconds, used for the animal to groom) [26].

2.2.2. Maternal behavior

To investigate the effects of SUL treatment during lactation on mother–pup interactions, two types of maternal behavior analyses were conducted. In experiment 1, pups were removed from their home cage, returned after 30 min and mother–pup interaction was observed for 30 min. In experiment 2, undisturbed mother and pup interaction was recorded for 6 h. The experiment 1 was conducted on PND 5 and the experiment 2 was conducted on PNDs 5 and 10. PNDs 5 and 10 were chosen because on the early time point (PND 5), lactation costs less energy and pups have limited motor capacity whereas on the second time point (PND 10) lactation is more energetically expensive and pups' motor activity is increased. All maternal behavior analyses were carried out between 8:00 a.m. and 2:00 p.m.

Experiment 1. Maternal behavior observations after pup removal.

Maternal behavior was evaluated on PND 5 in a subset of dams (10 dams/group). On the test day, all pups were removed from the home cage and the nest was destroyed. After 30 min, the pups were returned to the cage and mother–pup interaction was recorded for 30 min. Latency for retrieval behavior and total time grouping, pup grooming, self grooming, crouching, off pups (defined as the amount of time the rat spent without any kind of interaction with pups regardless of her position in the cage), and nest building were observed. Full maternal behavior was scored if dams retrieved all pups to the nest and nursed them for 3 consecutive minutes. All behavioral analyses were performed using Etholog software [27].

Experiment 2. Undisturbed mother–pup interaction observations.

Maternal behavior was evaluated on PNDs 5 and 10 in a subset of dams (10 dams/groups). Mother and litter interactions were recorded on their home cage for 6 h. Videotaping began at 08:00 a.m. in the light phase. Behavior was scored every minute during this period

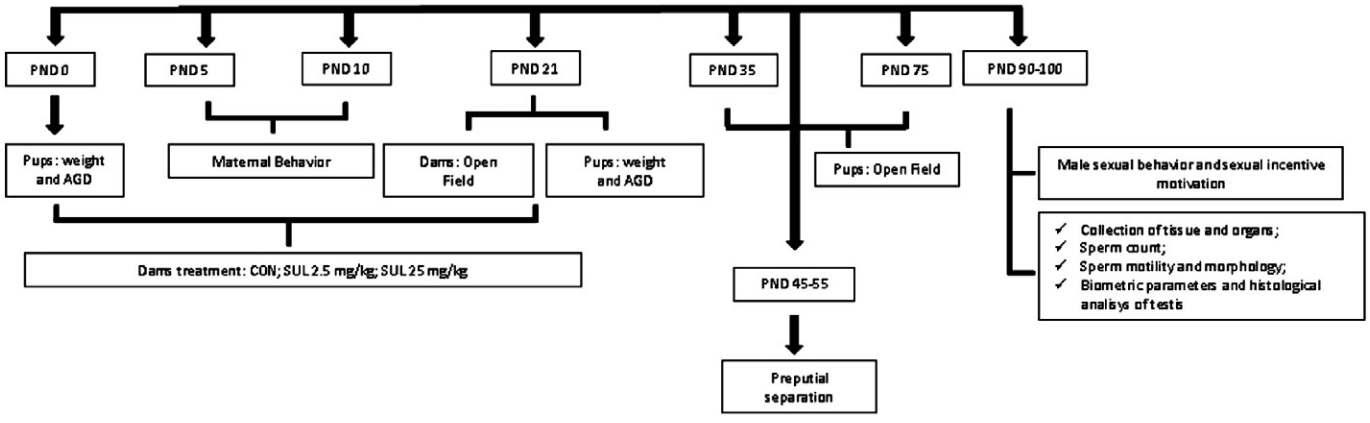

Fig. 1. Diagram of the experimental design. PND: postnatal day; AGD: anogenital distance; CON: distilled water; SUL 2.5: Sulpiride 2.5 mg/kg; SUL 25: Sulpiride 25 mg/kg.

according to the following categories: nursing, off pups, retrieval behavior, pup grooming and self-grooming. Total number of observations was used to calculate the percentage of observations in self-grooming, pup-grooming, nursing, and off pups.

2.3. Parameters analyzed in pups

2.3.1. Physical development of pups

Pups' body weights were measured weekly (PNDs 0, 7, 14, 21) during lactation. On PNDs 0 and 21, the anogenital distances (AGD, distance from the anus to the genital tubercle) were obtained through a vernier caliper. AGD was normalized through its division by the cube root of body weight. From PND 45, preputial separation of males was verified daily and considered as indicators of the sexual maturity onset. These data are expressed as litter mean.

2.3.2. General activity of pups

Male pups were submitted to behavioral analysis in the open-field on PNDs 35 and 75 according to the methodology described in Section 2.2.1, to evaluate if motor alterations that could interfere in the sexual behavior assessment were present after lactational exposure to SUL. The animals were used only once (i.e. different animals belonging to the same litter were used at each age) and all behavioral tests were recorded by a video camera, linked to a computer in an adjacent room. Videos were analyzed blindly to treatment.

2.3.3. Reproductive development in male pups

For the evaluation of male reproductive development, 2 pups (PNDs 90–100) from each litter were used, one for the sexual behavior evaluation and the other one for the sexual organ weights, sperm parameters and testis morphometry.

2.3.4. Collection of tissue and organs

One male rat per litter from each experimental group was euthanized with diethyl ether and the right testis and epididymis, ventral prostate and seminal vesicle (without the coagulating gland and full of secretion) were removed and their weights (absolute and relative to body weight) were determined. The right testis and epididymis were frozen at −20 °C for sperm counting. The left testis was collected for histopathological analysis.

2.3.5. Daily sperm production per testis, sperm number and transit time in the epididymis

Right testis was decapsulated and the caput/corpus and cauda segments from epididymis were separated (n = 15/group). Homogenization-resistant testicular spermatids (stage 19 of spermiogenesis) and sperm in the caput/corpus epididymis and cauda epididymis were assessed as described previously by Robb et al. [28], with adaptations of Fernandes et al. [29]. Mature spermatids were counted in a Neubauer chamber (four fields per animal). To calculate daily sperm production (DSP) number of spermatids at stage 19 was divided by 6.1, which is the number of days in one seminiferous cycle when these spermatids are present in the seminiferous epithelium. Sperm transit time through the epididymis was determined by dividing the number of sperm in each segment by the DSP.

2.3.6. Sperm motility and morphology

Immediately after euthanasia, sperm were obtained from the right vas deferens duct (n = 11–12/group) and diluted in 1 ml of modified HTF medium (Human Tubular Fluid, Irvine Scientific™), pre-warmed at 34 °C. A 10 μl aliquot was placed in a Makler chamber (Irvine) and analyzed under a phase-contrast microscope (OSM-223287, Olympus) at 100× magnification. One hundred sperm were evaluated per animal and classified for motility into: mobile and immotile [30]. With the aid of a syringe and a needle, sperm were recovered from the left vas deferens by flushing with 1.0 ml of saline formol (10%). To analyze the sperm morphologically, smears were prepared on histological slides that were left to dry for 90 min and 200 spermatozoa per animal were analyzed in a phase-contrast microscope (400× magnification) [31]. Morphological abnormalities were classified into two general categories: head morphology (without characteristic curvature or isolated form, i.e., no tail attached) and tail morphology (broken or isolated i.e., no head attached) [32].

2.3.7. Biometric parameters and histological analysis of testis

The left testis (n = 15/group) was promptly dissected, weighed and fixed by immersion in Bouin's solution for 24 h before being stocked in ethanol at 70 °C. Testes were cut into tissue fragments, dehydrated in increasing concentrations of ethanol, and embedded in paraffin. Blocks were sectioned at 5 μm and stained with hematoxylin–eosin.

For testis morphometry, the average diameter of thirty cross sections of round sex cord/seminiferous tubules per animal was obtained in a linear reticule micrometer (OSM-223287, Olympus) coupled to an ocular microscope with 100× final magnification. The volume densities of various testicular tissue components were determined by light microscopy using a 100-intersection grid placed in the ocular of the light microscope. Thirty fields chosen randomly (3000 points) were scored for each animal at 400× magnification. The volume of each component of the testis was determined as the product of the volume density and testis volume. Because the testis density is nearly 1.0 (~1.03–4), for subsequent morphometric calculations the testis weight was considered equal to testis volume [33]. To obtain a more precise measure of testis volume the testis capsule (~6.5%) was excluded from the testis weight. The total length of seminiferous tubule (meters) was obtained by dividing seminiferous tubule volume by the squared radius (R^2) of the tubule times the π value. Two hundred random tubular sections per animal in 3 nonconsecutive testis cross-sections were analyzed (400× magnification) to determine the percentage of seminiferous tubules with alterations.

2.3.8. Male sexual behavior

Sexual behavior was observed in adult rat (n = 20/group) during the dark phase of a reversed light/dark cycle, under dim red light. The animals were allowed a 15-day period of adaptation to the reversed light/dark cycle before the beginning of the evaluations. The observations always started 4 h after the onset of darkness and were recorded by a video camera, linked to a monitor in an adjacent room. For the copulatory behavior evaluation, each male was placed into a Plexiglas cage and, after 5 min, a female in natural estrus was introduced into the cage. During 30 min, the latencies and numbers of intromissions and ejaculations were observed as described previously [34]. If a male did not mount within 10 min, the evaluation was interrupted and repeated in another day. If the male failed again in the second evaluation, it was considered sexually inactive.

2.3.9. Sexual incentive motivation

The same animals evaluated for copulatory behavior were submitted to the sexual incentive motivation test [35]. In this test, a rectangular arena with 50 × 50 × 100 cm (height × width × length) that presents two openings that communicate with two small arenas with 25 cm^2 was used. The small arenas were diagonally opposed to each other and the communication with the main arena is closed with wire mesh. For the test, an estrous female (sexual incentive) was placed in one of the small arenas and a sexually active male (social incentive) was placed in the other one. The floor of the main arena had two 25 cm^2 divisions (zones) in front of each small arena opening, named sexual incentive and social incentive zones, respectively. The experimental male was placed in the center of the main arena and observed for 20 min. The number of visits and the total time spent visiting each zone were quantified, and a preference score was calculated as (time spent in female zone / total time spent in both incentive zones) × 100 [35].

2.4. Statistical analysis

Initially, an exploratory analysis was conducted to evaluate normal distribution (Shapiro–Wilk test) and homogeneity of variance (Levene's test) of each variable. Variables that presented normal distribution and homogeneity of variance were analyzed by ANOVA complemented with Bonferroni post hoc test. Conversely, for the other variables Kruskal–Wallis complemented with Dunn's test were performed. For dams and pups' weight, food intake, AGD and maternal behavior (undisturbed mother–pup interaction observations), repeated measures ANOVA (RMANOVA) was applied with day as the within-subject factor and treatment as the between-subjects factors. Differences were considered significant if $p < 0.05$.

3. Results

3.1. Parameters analyzed in dams

3.1.1. Maternal general toxicity

Body weight (Fig. 2A), food intake (Fig. 2B) and general activity in the open-field (data not shown) of dams during lactation were unaffected by SUL treatment ($p > 0.05$).

3.1.2. Maternal behavior

3.1.2.1. Maternal behavior observations after pup removal. ANOVA indicated lack of treatment effect for all the parameters evaluated, i.e. retrieval behavior, total time grouping, pup grooming, self grooming, crouching, off pups, and nest building (data not shown).

3.1.2.2. Undisturbed mother–pup interaction observations. RMANOVA showed a significant ($p < 0.05$) effect of lactational day on the percentage of observations in nursing [PND 5 (CON: 57.69 ± 3.97; SUL 2.5: 62.22 ± 3.27 and SUL 25: 61.67 ± 3.99)/PND 10 (CON: 53.50 ± 3.05; SUL 2.5: 49.29 ± 4.55 and SUL 25: 52.28 ± 2.04) n = 10 dams/group], with dams spending more time nursing their offspring on PND 5 than on PND 10. There was no main effect of treatment, i.e., SUL did not affect maternal behavior.

3.2. Pups

3.2.1. Physical development of pups

Body weight of pups (Fig. 3) during the first three weeks of age and relative AGD ($mm/g^{1/3}$) of male pups at birth (CON: 1.69 ± 0.04; SUL 2.5: 1.71 ± 0.04 and SUL 25: 1.77 ± 0.05) as well as on PND 21 (CON: 16.79 ± 0.32; SUL 2.5: 17.57 ± 0.33 and SUL 25: 17.35 ± 0.31) were not influenced by SUL exposure ($p > 0.05$) as indicated by RMANOVA (n = 20 litters/group).

SUL exposure also did not influence the day of preputial separation of males (CON: 48.18 ± 0.49; SUL 2.5: 47.08 ± 0.67 and SUL 25: 46.71 ± 0.48, n = 20 litters/group). No significant difference in the body weight was observed among the groups at the day in which preputial cleavage occurred (data not shown).

3.2.2. Behavioral evaluation of pups

The behaviors (ambulation, rearing and grooming) evaluated in the open-field at PNDs 35 and 75 were not influenced by exposure to SUL (data not shown).

3.2.3. Reproductive analysis of adult male offspring

Body weight and reproductive organ/gland weight of adult male pups are presented in Table 1. ANOVA complemented with Bonferroni indicated that lactational exposure to SUL 25 increased body weight in PNDs 90–100, increased absolute weight of prostate and reduced

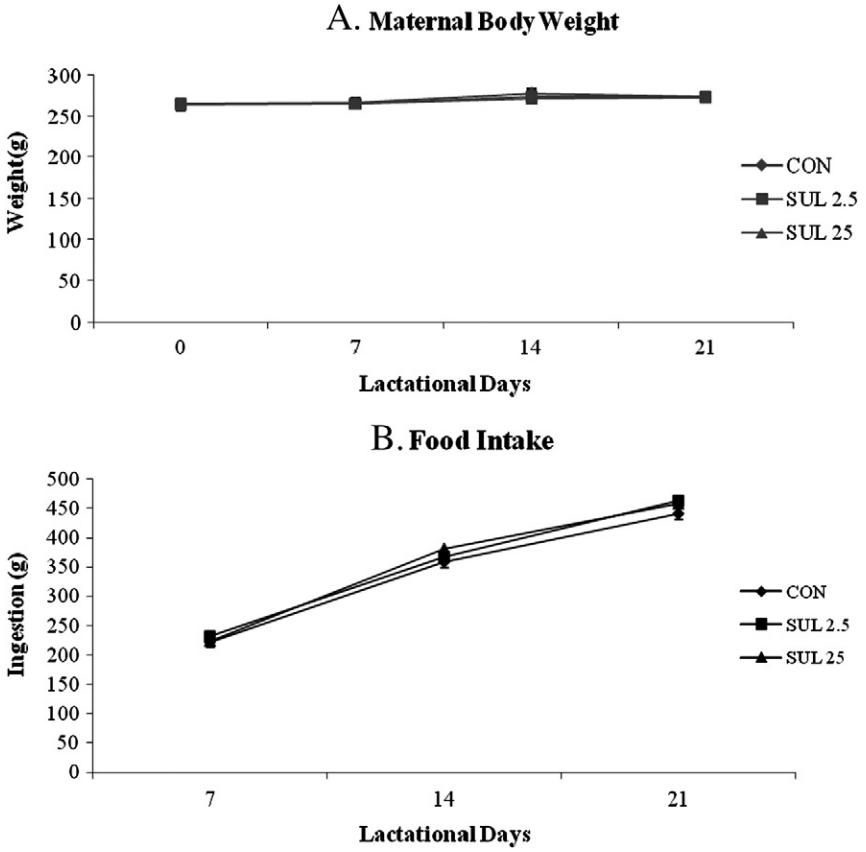

Fig. 2. Body weight (A) and food intake (B) of dams during lactation. Data are means ± SEM of 20 dams/group. ANOVA $p > 0.05$. CON: distilled water; SUL 2.5: Sulpiride 2.5 mg/kg; SUL 25: Sulpiride 25 mg/kg.

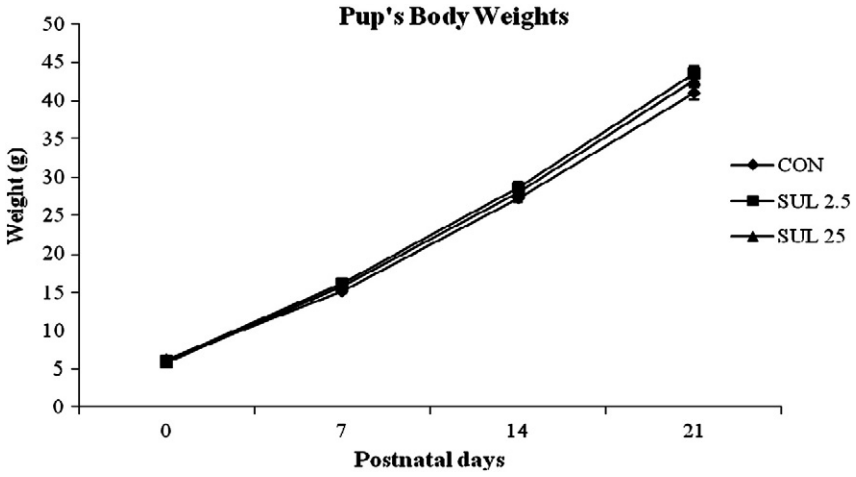

Fig. 3. Pups' body weights during the lactational period. Data are means ± SEM of 20 litters/group. ANOVA $p > 0.05$. CON: distilled water; SUL 2.5: Sulpiride 2.5 mg/kg; SUL 25: Sulpiride 25 mg/kg.

relative weight of testis ($p < 0.05$) when compared to CON group. In the SUL 2.5 exposure, ANOVA complemented with Bonferroni, indicated a decrease in relative and absolute weight of testis ($p < 0.05$) compared to CON. No statistical difference was observed in the other parameters.

In Table 2, sperm parameters are represented. SUL exposure did not alter sperm count in either doses (ANOVA, $p > 0.05$). However, an increase in percentage of abnormal head morphology sperm (ANOVA complemented with Bonferroni, $p < 0.05$) and in immotile sperm ($p < 0.05$, Kruskal–Wallis complemented with Dunn's test) in the SUL 25 group was observed compared to CON.

Biometric parameters of the testes are presented in Table 3. Kruskal–Wallis showed no effects of SUL exposure on the diameter and total length of the seminiferous tubules. On the other hand, Kruskal–Wallis showed a significant ($p < 0.05$) decrease in volume of seminiferous tubules in SUL 25-exposed rats and a trend ($p < 0.06$) towards reduction in the testicular volume in both doses. The histology of the seminiferous epithelium (Fig. 4) showed a significant increase ($p < 0.05$, Kruskal–Wallis complemented with Dunn's test) in the percentage of abnormal seminiferous tubules [CON: 0.00 (0.00–1.25); SUL 2.5: 2.00 (1.00–6.00)* and SUL 25: 5.00 (1.25–9.00)*] [% median (1–3 quartile), n = 15 animals/group]. The main testicular histological alteration observed in these animals was extensive desquamation of germ cells. Several seminiferous tubules showed severe immature germ cell loss in the group exposed to the highest dosage (SUL 25).

Table 1
Body weight and wet weight of organs from male rats at PNDs 90–100.

	CON (20)	SUL 2.5 (20)	SUL 25 (20)
Final body weight (g)	385.56 ± 8.00	385.47 ± 6.12	415.06 ± 7.14*
Absolute weights (g)			
Testis	1.56 ± 0.03	1.45 ± 0.02*	1.52 ± 0.08
Epididymis	0.52 ± 0.02	0.50 ± 0.01	0.54 ± 0.03
Prostate	0.36 ± 0.02	0.34 ± 0.02	0.44 ± 0.03*
Full seminal vesicle	0.64 ± 0.02	0.59 ± 0.03	0.65 ± 0.04
Empty seminal vesicle	0.19 ± 0.01	0.18 ± 0.01	0.19 ± 0.01
Relative weights (g/100 g)			
Testis	0.41 ± 0.01	0.38 ± 0.01*	0.37 ± 0.02 *
Epididymis	0.14 ± 0.005	0.13 ± 0.003	0.14 ± 0.006
Prostate	0.10 ± 0.01	0.09 ± 0.01	0.11 ± 0.01
Full seminal vesicle	0.17 ± 0.01	0.15 ± 0.01	0.16 ± 0.01
Empty seminal vesicle	0.05 ± 0.002	0.05 ± 0.003	0.05 ± 0.003

Data are means ± SEM. Numbers in parentheses represent the number of animals/group. CON: distilled water; SUL 2.5: Sulpiride 2.5 mg/kg; SUL 25: Sulpiride 25 mg/kg.
* $p < 0.05$ compared to CON (ANOVA complemented with Bonferroni).

Neither sexual behavior (Table 4) nor sexual motivation (Table 5) was altered by SUL exposure compared to CON group. Significant effects were observed between the two groups exposed to SUL in the latency to the first ejaculation, number of post-ejaculatory intromissions and number of ejaculations (Table 4). However, considering that SUL-exposed groups were not different from CON and the lack of a monotonic dose–response relationship, these results are suggested not to be biologically relevant.

4. Discussion

This study investigated the effects of lactational exposure to SUL, a dopaminergic antagonist, in maternal care and reproductive development of male pups. In reproductive and developmental toxicity studies, evaluation of maternal toxicity is critical and the main non-invasive indicators of maternal toxicity are the body weight gain and food intake [36]. In our study, SUL did not alter weight and food intake in dams during lactation. Although increased body weight was described in non-pregnant female rats [37,38], we believe that the lack of SUL-induced increase in body weight observed in this study with lactating dams is explained by the tremendous energy investment during lactation [39].

Mesolimbic and striatal dopaminergic pathways are thought to influence motor function such that DA receptor agonists stimulate and antagonists inhibit motor activity [25]. Although SUL is less potent than other antipsychotics such as haloperidol in inhibiting motor function [40], we decided to evaluate this parameter in dams as a biomarker of effect as well as a biomarker of general toxicity. The open field test showed that SUL treatment during lactation did not influence motor activity of dams at the end of lactation (PND 21). In the literature, SUL-induced motor function alterations only with higher doses (from 40 mg/kg) of SUL administered acutely to adult male rats are described [41,42].

Regarding maternal behavior in rat, its regulation occurs in two phases: onset, that occurs at parturition and is controlled by several pregnancy-related hormones (estrogen, progesterone, prolactin and oxytocin); and maintenance, that occurs during the postpartum period and is controlled primarily by nonhormonal factors (i.e., the multisensory stimuli provided by pups) [19]. Studies report that DA is involved in both the onset and the maintenance of maternal care [12–15]. In this study, SUL treatment during lactation did not impair mother–pup interaction and similar results were found with eticlopride, another D2 antagonist [43], although with different methodology. Other two studies reported disruption of retrieving after treatment with pimozide and clepobride, which are also D2 antagonists [19,20]. The lack of

Table 2
Sperm parameters of adult male rats at PNDs 90–100.

Parameters	CON	SUL 2.5	SUL 25
No. of spermatids (10^6/testis)	123.63 ± 2.75	113.49 ± 6.11	117.02 ± 7.09
No. of spermatids (10^6/g/testis)	83.56 ± 2.73	77.77 ± 4.45	79.88 ± 4.37
DSP	20.27 ± 0.45	18.28 ± 1.19	19.18 ± 1.16
No. of spermatozoa × 10^6/caput + corpus of epididymis	79.56 ± 5.15	76.25 ± 7.52	77.10 ± 9.73
No. of spermatozoa × 10^6/g/caput + corpus of epididymis	281.39 ± 24.18	286.71 ± 33.40	258.20 ± 28.82
No. of spermatozoa × 10^6/cauda of epididymis	122.35 ± 8.61	122.33 ± 5.33	121.56 ± 5.55
No. of spermatozoa × 10^6/g/cauda of epididymis	504.61 ± 38.61	508.16 ± 24.40	483.55 ± 33.11
Sperm transit time (days) through caput/corpus of epididymis	3.97 ± 0.26	4.50 ± 0.42	3.77 ± 0.43
Sperm transit time through cauda of epididymis (days)	6.09 ± 0.46	7.40 ± 0.90	6.60 ± 0.50
Abnormal head morphology sperm (%)	13.59 ± 1.50	15.56 ± 1.77	19.71 ± 1.63*
Mobile sperm (%)	84.00 (81.50–88.50)	75.00 (72.00–78.00)	72.00 (61.00–77.75)*
Immotile sperm (%)	16.00 (11.50–18.50)	25.00 (22.00–28.00)	26.50 (21.50–31.00)*

Data are means ± SEM of 15 animals/group for sperm counts and 11–12 animals/group for sperm morphology and motility. DSP: Daily sperm production. *$p < 0.05$ compared to CON (ANOVA complemented with Bonferroni).
Mobile and immotile sperm are presented as median (1°–3° quartile) and were analyzed by the non-parametric test of Kruskal–Wallis complemented with Dunn, *$p < 0.05$ compared to CON. CON: distilled water; SUL 2.5: Sulpiride 2.5 mg/kg; SUL 25: Sulpiride 25 mg/kg.

Table 3
Biometric parameters of testis from male rats at PNDs 90–100.

	CON (15)	SUL 2.5 (15)	SUL 25 (15)
Testicular volume (ml)	1.43 (1.37–1.53)	1.37 (1.27–1.48)	1.38 (1.28–1.42)
Volume of seminiferous tubules (ml)	1.12 (1.09–1.16)	1.03 (0.98–1.14)	1.02 (0.96–1.07)*
Diameter of seminiferous tubules (μm)	300.97 (279.89–308.95)	296.32 (287.94–304.40)	294.34 (283.49–305.01)
Total length of seminiferous tubules (m)	15.80 (15.20–17.72)	14.90 (13.60–16.90)	14.40 (12.90–16.95)

Numbers in parentheses represent the number of animals/group. Data are presented as median (1°–3° quartile) and were analyzed by the non-parametric test of Kruskal–Wallis complemented with Dunn, *$p < 0.05$. CON: distilled water; SUL 2.5: Sulpiride 2.5 mg/kg; SUL 25: Sulpiride 25 mg/kg.

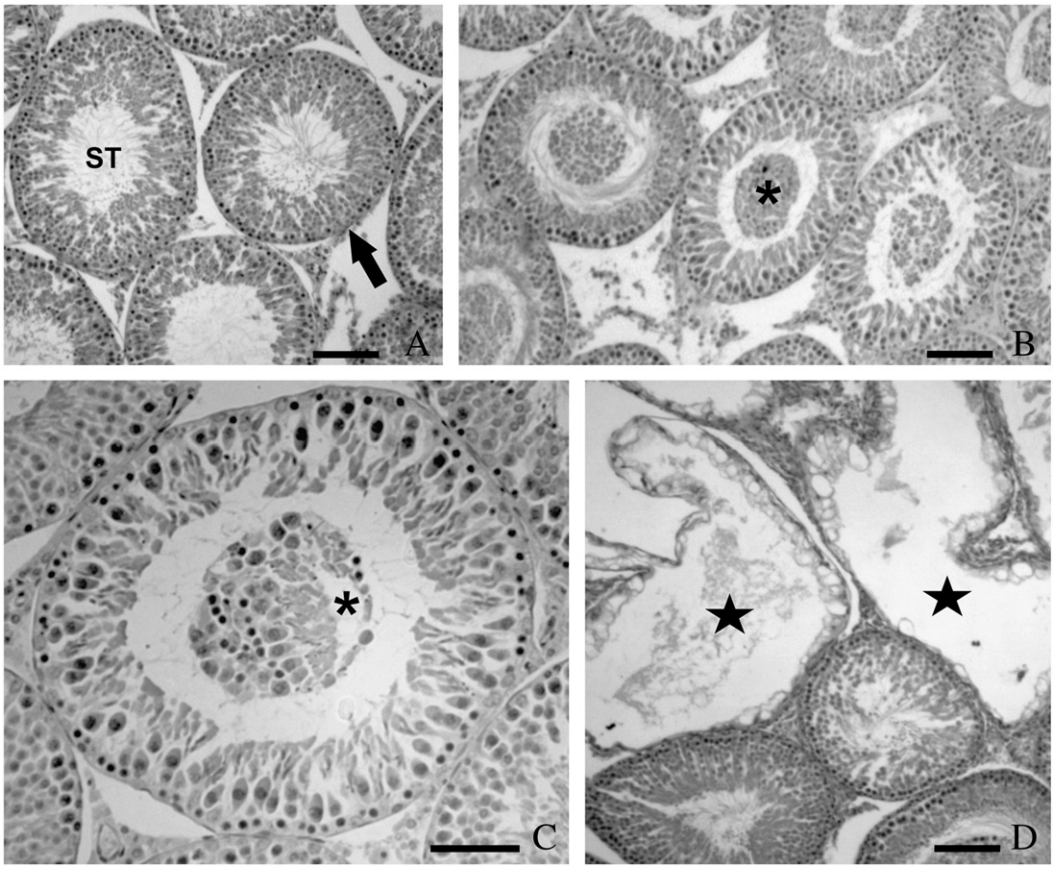

Fig. 4. Photomicrographs (histopathological analysis) of seminiferous tubule sections of rats from CON group (A) and groups exposed to SUL 2.5 (B and C) and SUL 25 (D), stained by HE. (A) Seminiferous tubules (TS) with normal aspect show germ cells organized in concentric layers constituting the seminiferous epithelium (arrow), (B and C) desquamation of immature germ cells (asterisks). (D) Observe the depleted seminiferous epithelium with severe germ cell loss (stars). Scale bars (A, B and D) = 10 μm, C = 50 μm. CON: distilled water; SUL 2.5: Sulpiride 2.5 mg/kg; SUL 25: Sulpiride 25 mg/kg.

Table 4
Sexual behavior of adult male rats at PNDs 90–100.

Parameters	CON	SUL 2.5	SUL 25
Latency to the first intromission (s)	175.06 ± 30.77 (16/20)	234.12 ± 32.33 (17/20)	146.82 ± 24.94 (17/20)
No. of intromissions until the first ejaculation	17.13 ± 1.58 (16/20)	20.53 ± 2.87 (17/20)	14.24 ± 1.42 (17/20)
Latency to the first ejaculation (s)	709.00 ± 80.61 (16/20)	784.76 ± 84.11 (17/20)	469.80 ± 48.16 (15/20)#
Latency of the first post-ejaculatory intromission (s)	328.64 ± 18.36 (15/20)	336.07 ± 18.25 (15/20)	300.33 ± 20.22 (15/20)
No. of post-ejaculatory intromissions	19.57 ± 2.16 (15/20)	14.40 ± 1.65 (15/20)	21.20 ± 1.23 (15/20)#
No. of ejaculations	2.20 ± 0.20 (15/20)	2.00 ± 0.15 (17/20)	2.73 ± 0.18 (15/20)#

Data are means ± SEM with the number of animals that displayed the behavior per total number of animals in the group given in parenthesis ($p < 0.05$, ANOVA completed with Bonferroni). #$p < 0.05$ compared to SUL 2.5. CON: distilled water; SUL 2.5: Sulpiride 2.5 mg/kg; SUL 25: Sulpiride 25 mg/kg.

consistency among the results may be explained by two different factors. First, pimozide also acts as an opioidergic receptor antagonist [44] and since the opioidergic system plays a role in maternal behavior [45], one could not assure that the disruption was attributed to the dopaminergic antagonism. Second, the different methodologies were employed, which mother and pups remained separated for a shorter period (15 min) [19,20] than the one used in this study (30 min). Since the longer the dam is separated from its pups the more motivated she becomes, longer periods of separation may overcome retrieving deficits [14,20].

Regarding pup evaluation, maternal exposure to SUL increased body weight of male offspring on PND 90 (highest dose), decreased both absolute (lowest dose) and relative (both doses) weights of testis, increased prostate absolute weight (highest dose), increased both the percentage of abnormal head morphology and the immobile sperm (highest dose), and decreased volume of seminiferous tubules in the testis (highest dose). Sperm production as well as motor and sexual behaviors were not influenced.

Persistent increased body weight has been described after SUL treatment of prepuberal [46] but not adult male rats [47]. Even though the authors discuss that this is an age-dependent effect in which gonadal steroids might be involved [46] the mechanism involved in SUL-induced weight gain after developmental exposure has not been investigated so far.

Among the parameters used in the evaluation of chemical risks to the male reproductive apparatus, is the determination of absolute and relative weights of endocrine organs/glands such as testes, epididymis, pituitary, seminal vesicle and prostate [48]. In the present study, the absolute weight of ventral prostate was increased by the highest dose of SUL but this increase was not detected when considering its relative weight. Since animals exposed to the highest dose of SUL presented increased body weight, one could infer that heavier animals presented heavier prostate absolute weight. However, it is noteworthy that if this was the case, other absolute organs/gland weights might have been increased as well, which did not happen. Moreover, increased prostate weights were described in male adults treated with SUL [49] and metoclopramide [50], another D2 antagonist. The authors suggested that hyperprolactinemia played a major role in this effect, since PRL is important in the regulation of normal growth and development of prostate, mainly in immature animals [51], acting as a local growth factor for the prostatic epithelium [52].

In the current study, testicular damage after SUL exposure was demonstrated by different parameters evaluated (reduced absolute weight and the volume of seminiferous tubules, negative influence on sperm parameters). PRL plays an important role in the regulation of testicular function in rodents [22] and humans [53] by affecting gonadotropin-releasing hormone (GnRH) output and, consequently modulating the luteinizing hormone (LH) released by the pituitary [54]. In rats, increased PRL levels are often associated with regression of the seminiferous epithelium [21,22], deformity of spermatozoa [55] and decrease in the epididymal sperm motility [56]. In humans, hyperprolactinemia affects seminal fluid quality causing spermiogenic arrest and impairment of sperm motility [57]. Based on these considerations, it is suggested that the testicular damage observed after lactational exposure to SUL may have resulted from increased PRL levels during reproductive development due to D2 antagonism induced by SUL. It is interesting to mention that adult rats [58,59] and humans [60,61] treated with antipsychotics presented hyperprolactinemia with reduced gonadal function [58–61].

Another explanation to the effects observed in the prostate as well as in the testis could be a direct action of SUL on the organs/glands. DA receptors are expressed not only in the pituitary, but also in some peripheral organs, mainly during the developmental period [62]. D2 receptors have been found in spermatozoa [63,64], seminiferous tubule [64] and rat prostate gland [49]. Although the role of these receptors is unknown [63], their presence indicates that they are a potential target for drugs [63].

In conclusion, the present study shows that maternal exposure to SUL during lactation may impact the reproductive development of male rats and the testes seem to be the main target organ at adulthood. The necessity of more researches investigating the influence of SUL and other galactagogues on the development of reproductive organs/glands in order to ensure the safety of their use is clear.

Table 5
Sexual incentive motivation test in male rats at PNDs 90–100.

	CON (20)	SUL 2.5 (20)	SUL 25 (20)
Time spent in male zone (s)	237.25 ± 27.88	299.75 ± 39.27	300.25 ± 24.21
Time spent in female zone (s)	568.20 ± 45.70	489.15 ± 41.61	516.60 ± 39.62
Number of visits in male zone	16.95 ± 1.40	19.15 ± 1.36	18.75 ± 1.26
Number of visits in female zone	20.75 ± 1.45	20.40 ± 1.20	20.00 ± 1.33
Preference score (%)	0.69 (61.51–84.76)	0.62 (54.76–76.66)	0.62 (55.67–73.50)

Numbers in parentheses represent the number of animals/group. Data are means ± SEM. ANOVA, $p > 0.05$. Preference score is presented as median (1°–3° quartile) and was analyzed by the non-parametric test of Kruskal–Wallis. CON: distilled water; SUL 2.5: Sulpiride 2.5 mg/kg; SUL 25: Sulpiride 25 mg/kg.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

This work was supported by PROIC/UEL (Scientific Initiation fellowship AHS and LSS) and CAPES (Master fellowship to MLV).

References

[1] Appendix P, Protocol # 9. Use of galactagogues in initiating or augmenting maternal milk supply. In: Lawrence RA, Lawrence RM, editors. Breastfeeding. A guide for the medical professional, 7th editionUSA: Elsevier; 2011. p. 1015–9.
[2] Amir LH. Breastfeeding — managing 'supply' difficulties. Aust Fam Physician 2006;35(9):686–9.
[3] Anderson PO, Valdés V. A critical review of pharmaceutical galactagogues. Breastfeed Med 2007;2(4):229–42.

[4] Mohameda GG, Solimanb MH. Synthesis, spectroscopic and thermal characterization of sulpiride complexes of iron, manganese, copper, cobalt, nickel, and zinc salts. Antibacterial and antifungal activity. Spectrochim Acta A 2010;76:341–7.
[5] Gabay MP. Galactogogues: medications that induce lactation. J Hum Lact 2002;18:274–9.
[6] Lasich AJ. Sulpiride and breastfeeding. Afr Med J 2005;95(9):624–6.
[7] Lewis M, Howie PW. Induction of transient hyperprolactinaemia in neonatal rats by direct or maternal treatment with the dopamine receptor blocker, sulpirida. Acta Endocrinol 1987;115:357–64.
[8] Aono T, Shioji T, Aki T, Hirota K, Nomura A, Kurachi K. Augmentation of puerperal lactation by oral administration of sulpiride. J Clin Endocrinol Metab 1979;48(3):478–82.
[9] Ylikorkala O, Kauppila A, Kivinen S, Viinikka L. Sulpiride improves inadequate lactation. Br Med J (Clin Res Ed) 1982;285(6337):249–51.
[10] Zuppa AA, Sindico P, Orchi C, Carducci C, Cardiello V, Romagnoli C, et al. Safety and efficacy of galactogogues: substances that induce, maintain and increase breast milk production. J Pharm Pharm Sci 2010;13(2):162–74.
[11] Schaefer C. Psychotropic drugs. In: Schaefer C, Peters PWJ, Miller RK, editors. Drugs during pregnancy and lactation, 2th edition — treatment options and risk assessment. United Kingdom: Academic Press; 2007. p. 708–37.
[12] Bridges RS, DiBiase R, Loundes DD, Doherty PC. Prolactin stimulation of maternal behavior in female rats. Science 1985;227:782–4.
[13] Bridges RS, Numan M, Ronshein PM, Mann PE, Lupini CE. Central prolactin infusions stimulate maternal behavior in steroid-treated, nulliparous female rats. Proc Natl Acad Sci U S A 1990;87(20):8003–7.
[14] Hansen S. Maternal behavior of female rats with 6-OHDA lesions in the ventral striatum: characterization of the pup retrieval deficit. Physiol Behav 1994;55:615–20.
[15] Lonstein JS, Simmons DA, Swann JM, Stern JM. Forebrain expression of c-fos due to active maternal behaviour in lactating rats. Neuroscience 1998;82:267–81.
[16] Numan M, Fleming AS, Levy F. Maternal behavior. In: Neil JD, et al, editor. Knobil and Neill's physiology of reproduction. 3th edition. USA: Elsevier; 2005. p. 1921–93.
[17] Numan M. Neural basis of maternal behavior in the rat. Psychoneuroendocrinology 1988;13(1–2):47–62.
[18] Stolzenberg DS, Numan M. Hypothalamic interactions with the mesolimbic DA system in the control of the maternal and sexual behaviors in rats. Neurosci Biobehav Rev 2011;35(3):826–47.
[19] Silva MR, Bernardi MM, Felicio LF. Effects of dopamine receptor antagonists on ongoing maternal behavior in rats. Pharmacol Biochem Behav 2001;68(3):461–8.
[20] Byrnes EM, Rigero BA, Bridges RS. Dopamine antagonists during parturition disrupt maternal care and the retention of maternal behavior in rats. Pharmacol Biochem Behav 2002;73(4):869–75.
[21] Mishra AC, Mohanty B. Lactational exposure to atypical antipsychotic drugs disrupts the pituitary-testicular axis in mice neonates during post-natal development. J Psychopharmacol 2010;24(7):1097–104.
[22] Jedlinska M, Rozewiecka L, Ziecik AJ. Effect of hypoprolactinaemia and hyperprolactinaemia on LH secretion, endocrine function of testes and structure of seminiferous tubules in boars. J Reprod Fertil 1995;103:265–72.
[23] Hallbauer U. Sulpiride (Eglonyl)-use to stimulate lactation. S Afr Med J 1997;87(6):774–5.
[24] Broadhurst PL. Experiments in psychogenetics. In: Eysenk HJ, editor. Experiments in personality. London: Rutledge and Kegan Paul; 1960. p. 31–61.
[25] O'Connor SE, Brown RA. The pharmacology of sulpiride — a dopamine receptor antagonist. Gen Pharmacol 1982;13(3):185–93.
[26] Moreira EG, Vassilieff I, Vassilieff VS. Developmental lead exposure: behavioral alterations in the short and long term. Neurotoxicol Teratol 2001;23:489–95.
[27] Ottoni EB. EthoLog 2.2: a tool for the transcription and timing of behavior observation sessions. Behav Res Methods Instrum Comput 2000;32(3):446–9.
[28] Robb GW, Amann RP, Killian GJ. Daily sperm production and epididymal sperm reserves of pubertal and adult rats. J Reprod Fertil 1978;54(1):103–7.
[29] Fernandes GS, Arena AC, Fernadez CD, Mercadante A, Barbisan LF, Kempinas WG. Reproductive effects in male exposed to diuron. Reprod Toxicol 2007;23(1):106–12.
[30] Perobelli JE, Martinez MF, Franchi CAS, Fernandez CDB, Camargo JLV, Kempinas WG. Decreased sperm motility in rats orally exposed to single or mixed pesticides. J Toxicol Environ Health A 2010;73(13):991–1002.
[31] Seed J, Chapi RE, Clegg ED, Dostal LA, Foote RE, Hurtt ME, et al. Methods for assessing sperm motility, morphology, and counts in the rat, rabbit, and dog: a consensus report. Reprod Toxicol 1996;10(3):237–44.
[32] Filler R. Methods for evaluation of rats epididymal sperm morphology. In: Chapin RE, Heindel JH, editors. Male reproductive toxicology. San Diego, CA: Academic Press Inc.; 1993. p. 334–43.
[33] França LR, Godinho CL. Testis morphometry, seminiferous epithelium, cycle length, and daily sperm production in domestic cats (*Felis catus*). Biol Reprod 2003;68:1554–61.
[34] Gerardin DCC, Bernardi MM, Moreira EG, Pereira OCM. Neuroendocrine and reproductive aspects of adult male rats exposed neonatally to an antiestrogen. Pharmacol Biochem Behav 2006;83:618–23.
[35] Agmo A. Lack of opioid or dopaminergic effects on unconditioned sexual incentive motivation in male rats. Behav Neurosci 2003;117:55–68.
[36] Bacchi AD, Ponte B, Vieira ML, De Paula JCC, Mesquita SFP, Gerardin DCC, et al. Developmental exposure to *Passiflora* incarnata induces behavioral alterations in the male progeny. Reprod Fertil Dev 2013;25(5):782–9.
[37] Baptista T, de Baptista EA, Lalonde J, Plamondon J, Kin NM, Beaulieu S, et al. Comparative effects of the antipsychotics sulpiride and risperidone in female rats on energy balance, body composition, fat morphology and macronutrient selection. Prog Neuropsychopharmacol Biol Psychiatry 2004;28(8):1305–11.
[38] Parada MA, Hernandez L, Paez X, Baptista T, Puig de Parada M, de Quijada M. Mechanism of the body weight increase induced by systemic sulpiride. Pharmacol Biochem Behav 1989;33(1):45–50.
[39] Woodside B, Augustine RA, Ladyman SR, Naef L, Grattan DR. Role of prolactin in the metabolic adaptations to pregnancy and lactation. In: Bridges RS, editor. Neurobiology of the parental mind. USA: Elsevier; 2008. p. 249–68.
[40] Costall B, Naylor RJ. Antagonism of the hyperactivity induced by dopamine applied intracerebrally to the nucleus accumbens septi by typical neuroleptics and by clozapine, sulpiride and thioridazine. Eur J Pharmacol 1976;35:161–8.
[41] Asmakova LS, Kalinina TS, Ostrovskaya RU, Gudasheva TA, Zaitseva NI, Bondarenko NA, et al. Comparison of antipsychotic activity and discriminative stimulus effects of the novel acylprolyltyrosine containing compound, GZR-123, and sulpiride. Pharmacol Biochem Behav 1999;64(2):359–62.
[42] Bruhwyler J, Chleide E, Liégeois JF, Delarge J, Mercier M. Anxiolytic potential of sulpiride, clozapine and derivatives in the open-field test. Pharmacol Biochem Behav 1990;36(1):57–61.
[43] Numan M, Numan MJ, Pliakou N, Stolzenberg DS, Mullins OJ, Murphy JM, et al. The effects of D1 or D2 dopamine receptor antagonism in the medial preoptic area, ventral pallidum, or nucleus accumbens on the maternal retrieval response and other aspects of maternal behavior in rats. Behav Neurosci 2005;119(6):1588–604.
[44] Friedman JI, Lindenmayer JP, Alcantara F, Bowler S, Parak M, White L, et al. Pimozide augmentation of clozapine in patients with schizophrenia and schizoaffective disorder unresponsive to clozapine monotherapy. Neuropsychopharmacology 2011;36(6):1289–95.
[45] Teodorov E, Tomita AT, Banon GP, Gil IG, Bernardi MM, Felício LF. Behavioral effects of acute stimulation of kappa-opioid receptors during lactation. Pharmacol Biochem Behav 2008;90(4):534–9.
[46] Baptista T, Parada MA, Murzi E. Puberty modifies sulpiride effects on body weight in rats. Neurosci Lett 1988;92:161–4.
[47] Baptista T, Parada M, Hernandez L. Long term administration of some antipsychotic drugs increases body weight and feeding in rats. Are D2 dopamine receptors involved? Pharmacol Biochem Behav 1987;27(3):399–405.
[48] Clegg ED, Perreault D, Klinefelter GR. Assessment of male reproductive toxicity. In: Hayes AW, editor. Principles and methods of toxicology. Philadelphia: Taylor & Francis; 2001. p. 1263–300.
[49] Sánchez P, Torres JM, Vílchez P, Del Moral RG, Ortega E. Effects of sulpiride on mRNA levels of steroid 5alpha-reductase isozymes in prostate of adult rats. IUBMB Life 2008;60(1):68–72.
[50] Sánchez P, Torres JM, Castro B, Frías JF, Ortega E. Effects of metoclopramide on mRNA levels of steroid 5α-reductase isozymes in prostate of adult rats. J Physiol Biochem 2013;69(1):133–40.
[51] Costello LC, Franklin RB. Effect of prolactin on prostate. Prostate 1994;24:162–6.
[52] Crepin A, Bidaux G, Vanden-Abeele F, Dewailly E, Goffin V, Prevarskaya N, et al. Prolactin stimulates prostate cell proliferation by increasing endoplasmic reticulum content due to SERCA 2b over-expression. Biochem J 2007;401:49–55.
[53] Cheung CY. Prolactin suppresses luteinizing hormone secretion and pituitary responsiveness to luteinizing hormone–releasing hormone by a direct action at the anterior pituitary. Endocrinology 1983;113:632–8.
[54] Henderson HL, Townsend J, Tortonese DJ. Direct effects of prolactin and dopamine on the gonadotroph response to GnRH. J Endocrinol 2008;197(2):343–50.
[55] Laszczyńska M, Rózewicka L, Kuchnio M, Piasecka M, Marchlewicz M. Evaluation of spermatozoa of rat in hyperprolactinaemia induced by metoclopramide. Andrologia 1992;24(2):101–8.
[56] Huang WJ, Wang PS, Yeh JY. The effect of hyperprolactinemia on epididymal sperm motility in rats. Fertil Steril 2008;90:S313.
[57] De Rosa M, Colao A, Di Sarno A, Ferone D, Landi ML, Zarrilli S, et al. Cabergoline treatment rapidly improves gonadal function in hyperprolactinemic males: a comparison with bromocriptine. Eur J Endocrinol 1998;138(3):286–93.
[58] Doherty PC, Baum MJ, Todd RB. Effects of chronic hyperprolactinemia on sexual arousal and erectile function in male rats. Neuroendocrinology 1986;42:368–75.
[59] Zhang X, Zhang Z, Cheng W, Mou X, Reynolds GP. The effect of chronic antipsychotic treatment on sexual behaviour, hormones and organ size in the male rat. J Psychopharmacol 2007;21(4):428–34.
[60] Yazigi RA, Quintero CH, Salameh WA. Prolactin disorders. Fertil Steril 1997;67:215–25.
[61] Knegtering M, Van der Moolen AE, Castelein S, Kluiter H, Van den Bosch RJ. What are the effects of antipsychotics on sexual dysfunctions and endocrine functioning? Psychoneuroendocrinology 2003;28:109–23.
[62] Saifetyarova YY, Sapronova AY, Ugryumov MV. Endocrine function of dopaminergic neurons of the whole rat brain in ontogeny: control of prolactin secretion. Dokl Biol Sci 2012;443(6):753–6.
[63] Ramírez AR, Castro MA, Angulo C, Ramió L, Rivera MM, Torres M, et al. The presence and function of dopamine type 2 receptors in boar sperm: a possible role for dopamine in viability, capacitation, and modulation of sperm motility. Biol Reprod 2009;80:753–61.
[64] Otth C, Torres M, Ramírez A, Fernandez JC, Castro M, Rauch MC, et al. Novel identification of peripheral dopaminergic D2 receptor in male germ cells. J Cell Biochem 2007;100(1):141–50.